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| REPORT DOCUMENTATION PAGE | | | Form Approved OMB No. 0704-0188 | |
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503. | | | | |
| 1. AGENCY USE ONLY (Leave blank) | | 2. REPORT DATE March 30, 2001 | | 3. REPORT TYPE AND DATES COVERED Final Report- 18-SEPT-96 to 19-SEPT-99 |
| 4. TITLE AND SUBTITLE Novel Near Infrared Fluorescent Dyes | | | 5. FUNDING NUMBERS N00014-96-C-0378 | |
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| 8. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy St. Arlington, VA 22217-5000 | | | 10. SPONSORING/MONITORING AGENCY REPORT NUMBER | |
| 11. SUPPLEMENTARY NOTES | | | | |
| 12a. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited | | | 20010409 049 | |
| 13. ABSTRACT (Maximum 200 words) The overall objective of this program is to produce new fluorophores suitable for biosensor signal transduction with excitation wavelengths greater than 665 nm and having electrophilic functionalities for covalent attachment to proteins and nucleic acids. The Phase I effort demonstrated the synthesis of representative fluorophores and characterized their fluorescence emission spectra. The main approach was to synthesize novel styryl dyes with features that result in long wavelength absorbance and emission spectra. The results obtained in the Phase I provide a rationale to achieve specific bathochromic shifts. This approach will be implemented in the Phase II effort to develop a series of dyes. To date we have a) demonstrated the feasibility of the proposed synthetic approaches to design long-wavelength styryl dyes, b) have prepared a series of dyes with long wavelength absorption, high quantum yield and large extinction coefficients, c) prepared derivatives of styryl dyes with succinimydyl ester groups, and d) demonstrated the feasibility of using these derivatives for covalent labeling of biological macromolecules. | | | | |
| 14. SUBJECT TERMS Fluorescence, Biosensor, Near Infrared, Fluorophores, Styryl, Synthesis, Spectral, Laser-Diode | | | 15. NUMBER OF PAGES 4 | |
| | | | 16. PRICE CODE | |
| 17. SECURITY CLASSIFICATION OF REPORT Unclassified | 18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified | 19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified | 20. LIMITATION OF ABSTRACT UL | |

FINAL REPORT

CONTRACT NUMBER: N00014-96-C-0378

PRINCIPAL INVESTIGATOR: Ernest F. Guignon

INSTITUTION: Ciencia, Inc.

CONTRACT TITLE: Novel Near Infrared Fluorescent Dyes

AWARD PERIOD: 18 September 1996 ☒ 19 September 1999.

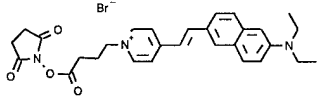
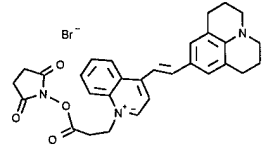
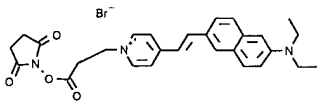
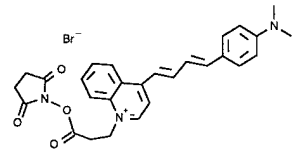
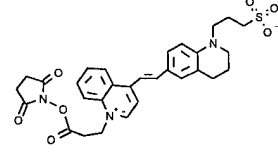
OBJECTIVE: To produce new functionalized fluorophores suitable for biosensor signal transduction with excitation wavelengths greater than 665 nm for covalent attachment to proteins and nucleic acids.

APPROACH: To synthesize new styryl dyes with features that produce large bathochromic shifts in their absorption and emission spectra, and with electrophilic side chains for covalent labeling of biomolecules. Styryl dyes possess many favorable characteristics such as large Stokes' shifts of 150 to 250 nm, large extinction coefficients of up to 5×10^4 , good photostability, and high quantum yields when immobilized in biological membranes.

ACCOMPLISHMENTS: Progress in the synthesis of styryl dyes has been significant during the past 8 months and can be divided into two related parallel efforts. We have prepared two new styryl reagents with succinimidyl ester groups for labeling amino side chains in proteins. For one of these, KDH137, a conjugate to IgG has been prepared. This conjugate displays a broad absorbance centered around 600nm and an emission maximum at 683nm; the dye to protein ratio for the conjugate was ca. 2:1. Second, we have prepared three new chromophores containing thiophene linkers to extend the conjugation within the polyene chain between the donor and acceptor moieties. The longest absorbance maximum of these three occurs at 670nm in ethanol for JPW3067 and we expect all of these to be further red-shifted in the protein-conjugated versions when the succinimidyl esters are synthesized. Even more remarkable is the extraordinary Stokes shift exhibited by these new dyes. For dye JPW3066, the Stokes

shift in ethanol is 322nm ($\lambda_{abs}=602\text{nm}$; $\lambda_{em}=924\text{nm}$). The dyes also have the large environmental sensitivity typical of the styryls: for JPW3066, the absorbance maximum shifts 60 nm to the red in ethanol compared to water and the fluorescence displays a huge enhancement upon binding to biological assemblies such as lipid bilayers.

Properties of Selected Styryl Fluorescent Reagents

| Name | Chromophore | Description | ABS (nm) | EM (nm) | Structure |
|---------|--------------------------------|---------------------------------------------------------------------------------------------------------------------------------|----------|---------|---------------------------------------------------------------------------------------|
| KDH10 | naphthylstyryl | Reagent for covalently labelling free amino groups on biomolecules. | 497 | 700 |  |
| KDH137 | styryl | Succinimidyl ester with quinolinium and julolidine features in chromophore. Conjugation to IgG gives abs at 609nm, em at 682 nm | 610 | |  |
| JPW3045 | Naphthylstyryl | Conjugation to IgG gives ex at 564nm, em at 680 nm | 500 | 720 |  |
| KDH187 | styryldienylquinolinium | succinimidyl ester quinolinium diene dimethylaniline - conjugatable | 540 | 670 |  |
| KDH239B | dihydroquinolinenylquinolinium | hydrophilic conjugatable structure with quinolinium and dihydroquinoline | 573 | 688 |  |

CONCLUSIONS: We have succeeded in developing synthetic routes to all the main target molecules proposed in our grant application as well as some new compounds that were suggested by our studies. These include new long-wavelength thiophene and furan containing analogs of the simple styryl chromophores. Comparison of analogous thiophene and furan dyes gives the surprising result that the furan dyes absorb light at longer wavelengths. The protein-conjugatable thiophene centered dye 1-[3'-N-hydroxy-succinimidylcarboxyethyl]-2-[p-(dimethylamino)styryl]thiophenyl-5-vinyl-4-quinolinium iodide

absorbs light at 593 nm in ethanol while the furan centered dye absorbs light at 612 nm. 1-(3-N-Trimethylammoniopropyl)-2-[p-(dimethylamino)styryl]thiophenyl-5-vinyl-4-quinolinium dibromide absorbs light at 602 nm in ethanol while the furan derivative absorbs at 630 nm in ethanol. Further red shifts can be attained through the incorporation of the julolidine moiety and through the replacement of the simple furan with a benzofuran.

SIGNIFICANCE: Most fiber-optic biosensors have been limited to the use of ultraviolet (UV) or visible dyes. These dyes possess good quantum yields, are commercially available with a functionality for covalent conjugation, and can be excited with the 488 nm line of an argon laser. However, argon lasers are bulky, costly and inefficient; excitation in the visible induces considerable non-specific background fluorescence which decreases the detection limit, and high scattering limits the transmission distance over fiber-optics. Such systems are not well-suited for practical in situ applications.

These shortcomings can potentially be obviated with the use of near-infrared (NIR) probes. An obvious advantage of NIR probes is that they can be excited with inexpensive diode lasers (~\$30 compared to ~\$5K for an argon laser) or LEDs (~\$1) which are durable, small, light, rugged, and efficient (100 mW wall plug power vs. kW for an argon laser). Diode lasers and LEDs also can be directly modulated up to GHz frequencies, permitting implementation of frequency-domain detection system for fluorescence lifetime-based biosensors.

Additionally, NIR excitation reduces non-specific fluorescence background as compared to UV or visible excitation, and significantly reduces Rayleigh and Raman scattering. This improves sensitivity and permits transmission over greater lengths of optical fiber.

PATENT INFORMATION: Provisional patents have been filed for some of the structures developed.

PUBLICATIONS AND ABSTRACTS:

Loew, L. M. 1999. Potentiometric dyes and imaging membrane potential in single cells. In *Fluorescent and luminescent probes for biological activity*. W. T. Mason, editor. Academic Press, London. 210-221.